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The present invention relates to oligonucleotides and method for detecting methicillin-resistant *Staphylococcus aureus* (MRSA) in clinical examination. The oligonucleotides provided in the present invention are useful as reagents for genetic diagnosis which involves procedures such as cleavage, amplification and detection of RNA or DNA, and as reagents for inhibiting reverse transcription or translation of RNA. In particular, the sequences of the oligonucleotides provided in the present invention are useful for reagents or the like for quantitative determination and diagnosis of MRSA.

Prior Art

MRSA is a resistant strain of *Staphylococcus aureus* exhibiting resistance against β -lactamase-resistant penicillins, including methicillin, which are stable against β -lactamase produced by *Staphylococcus aureus*. MRSA is a major pathogen in the nosocomial infections, and strains thereof which exhibit a slight resistance even to vancomycin, an effective therapeutic drug against the resistant strain, have also been detected. MRSA is causing significant problems in medical care due to the lack of an effective antimicrobial drug thereto. Thus, its accurate and rapid detection in clinical examination is an important subject in diagnosis and treatment.

30 *Staphylococcus aureus* generally produces four types
of cell wall composing-proteins PBPs (penicillin-binding
proteins), i.e., PBP-1 through PBP-4, however, it had
been found that MRSA also produces a new type of PBP
35 termed PBP-2'. This type of PBP is a specific protein
having poor affinity against β -lactam antibiotics, and is

known to play a central role in the tolerance of this organism. The sequence of the *mecA* gene that codes for PBP-2' is known (FEBS Lett., 221, 167-171, 1987, etc.). Therefore, hybridization methods employing a gene probe
5 specific to *mecA* gene have been sought to detect and identify MRSA.

As mentioned above, although attempts have been made to detect MRSA at the gene level, since preparation of a sample requires culturing the bacteria obtained from a
10 patient specimen, these methods have problems in terms of their speed. Since detection and identification of MRSA requires long culturing period, and it is difficult to rapidly detect a trace amount of *mecA* gene in a sample, there is a need for the development of a rapidly and
15 highly sensitive detection method in the field of clinical diagnosis. Moreover, there is also a need to develop an automated examination apparatus to simplify examinations.

In order to carry out highly sensitive detection, it
20 is preferable to perform said detection after amplifying a specific sequence in the gene to be detected and identified, or in RNA derived from said gene (hereafter, referred to as the "target nucleic acid").

When the target nucleic acid is DNA, the Polymerase
25 chain reaction (PCR) method is known as an amplification method. This method amplifies a specific sequence by repetition of a cycle comprising heat denaturation, primer annealing and extension reactions, in the presence of a pair of primers complementary and homologous to both
30 ends of said specific sequence in the target DNA as well as a thermostable DNA polymerase. At this time, in order to amplify said specific sequence by PCR, oligonucleotides that are highly specific to said
35 specific sequence are required. Moreover, in order to carry out this detection and identification with high sensitivity, oligonucleotides that are highly specific to the target DNA are required. Further, it is also

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Thus, the *mecA* gene can be detected without culturing the bacteria from a specimen, making this method useful for rapid diagnosis. At this time, in the amplification of the above specific sequence by the NASBA method or the like, an oligonucleotide having high specificity to the above specific sequence is required. Moreover, in order to perform detection and identification with high sensitivity, an oligonucleotide that has high specificity to the target RNA is required. Therefore, an attempt to detect the *mecA* gene located on the chromosomal DNA of *Staphylococcus aureus* by the NASBA method using specific oligonucleotide sequences has been made. However, since the NASBA method or the like involves amplification reaction at relatively low temperature (for example, 41°C), the target RNA forms an intramolecular structure which inhibits binding of the primer, and may reduce the reaction efficiency. Consequently, a procedure of heat denaturation of the target RNA prior to the amplification reaction was required to break down the intramolecular structure of the target RNA, thereby to improve the primer binding efficiency. In addition, even in the case where detection of RNA is carried out at a low temperature, an oligonucleotide capable of binding to RNA which has formed the above-mentioned intramolecular structure is required.

Therefore, the first object of the present invention is to provide oligonucleotides that are useful in specific cleavage, amplification, or the like, as well as highly sensitive detection and identification of the mecA gene coding for cell wall composing-protein PBP-2' produced by MRSA or RNA derived from said gene. In addition, the present invention provides oligonucleotide sequences useful in a pharmaceutical composition for inhibiting RNA reverse transcription or translation.

The second object of the present invention is to provide a preferable combination of oligonucleotides useful in specific amplification of RNA derived from the *mecA* gene coding for cell wall composing-protein PBP-2' produced by methicillin-resistant *Staphylococcus aureus* at a relatively low temperature (e.g., 41°C), as well as for the highly sensitive detection and identification thereof.

Detailed Description of the Invention

The invention of claim 1, which has been accomplished to achieve the first object, relates to an oligonucleotide for cleavage, detection or amplification of the *mecA* gene, a gene element of methicillin-resistant *Staphylococcus aureus* (MRSA), or RNA derived from said gene, which oligonucleotide is capable of binding specifically to said *mecA* gene or RNA derived therefrom, and comprises at least 10 contiguous bases of any of the sequences listed as SEQ. ID. Nos. 1 to 17, or an oligonucleotide complementary to said oligonucleotide.

The invention of claim 2, which has been accomplished to achieve the aforementioned object, relates to the oligonucleotide according to claim 1, wherein said oligonucleotide is an oligonucleotide primer for DNA elongation reaction.

The invention of claim 3, which has been accomplished to achieve the aforementioned object, relates to the oligonucleotide according to claim 1, wherein said oligonucleotide is an oligonucleotide probe a portion of which is modified or labeled with a detectable marker.

The invention of claim 4, which has been accomplished to achieve the aforementioned object, relates to the oligonucleotide according to claim 3, wherein said oligonucleotide is a synthetic oligonucleotide in which a portion of its base(s) is(are) modified without impairing the function of said oligonucleotide as an oligonucleotide probe.

5 The oligonucleotides of the invention, which have
been accomplished to achieve the first object, are
oligonucleotides that complementarily bind in a specific
manner to intramolecular structure-free regions of the
target RNA in the aforementioned RNA amplification, and
they are capable of binding specifically to the target
RNA without the heat denaturation described above. In
this manner, the present invention provides
oligonucleotides that binds to intramolecular structure-
free regions of the RNA derived from the *mecA* gene coding
for PBP-2' at a relatively low and constant temperature
(35-50°C, and preferably 41°C), which are useful for
specific cleavage, amplification, detection or the like
of the *mecA* gene. More specifically, the present
invention relates to oligonucleotides which accomplish
rapidly and highly sensitive detection by their use as
oligonucleotide primers for amplifying the above target
DNA with PCR, oligonucleotide primers for amplifying the
above target RNA with NASBA or the like, and an
oligonucleotide probe for detecting the target nucleic
acid without or after these amplifications. In this
connection, recently, in order to improve genetic
detection technology, developments of new chemically
synthesized substances, which would not impair the
function of an oligonucleotide probe to recognize a
complementary sequence based on adenine, guanine,
cytosine and thymine (or uracil) bases have been carried
out. One example of such substances includes peptide
nucleic acid (PNA) in which the sugar and phosphoric acid
skeletons that provides the skeleton structure of nucleic
acid DNA have been replaced with a polyamide skeleton.
Thus, oligonucleotides that have been modified by a
substance such as PNA to an extent that would not impair
their base sequences recognizing-function are also
included in the detecting probes of the present
invention.

SEQ ID Nos.1 through 17 illustrate the

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oligonucleotides of the present invention useful in cleavage, amplification, detection or the like of RNA derived from the *mecA* gene. In this connection, RNA derived from the *mecA* gene also includes RNA that has been produced by using these genes as templates. Although each of the oligonucleotides of the present invention may include entire base sequence of any of SEQ ID Nos. 1 to 17, since an order of 10 bases is adequate for specific binding to *mecA* gene, these oligonucleotides can be oligonucleotides comprising at least 10 contiguous bases of the described sequences, and may also be their complementary oligonucleotides.

The oligonucleotides of the present invention can be used, for example, as an oligonucleotide primer for nucleic acid amplification. If a nucleic acid amplification method is carried out using the oligonucleotide of the present invention as the primer, only the target nucleic acid, namely *mecA*, can be amplified. Although examples of amplification methods include PCR, LCR, NASBA and 3SR, nucleic acid amplification methods that can be carried out at a constant temperature such as LCR, NASBA and 3SR are particularly preferable. MRSA can be detected by detecting the amplification product by various methods. In this case, any of the above oligonucleotides other than the oligonucleotide used in the amplification may be used as the probe, and the fragment of the amplified specific sequence can be confirmed by electrophoresis or the like.

The oligonucleotides of the present invention can be used as probes by, for example, modifying portions or labeling them with a detectable marker. When detecting the target nucleic acid, the oligonucleotide of the present invention labeled with the detectable marker may be hybridized to a single-stranded target nucleic acid, after which the hybridized probe can be detected via the marker. The marker detection may be carried out by a

method suitable for the particular marker and, for example, when using an intercalator fluorescent dye for labeling the oligonucleotide, a dye with the property of exhibiting increased fluorescent intensity by
5 intercalation in the double-stranded nucleic acid comprising the target nucleic acid and the oligonucleotide probe may be used in order to allow easy detection of only the hybridized probe without removal of the probe that has not hybridized to the target nucleic
10 acid. When using a common fluorescent dye as the marker, the marker may be detected after removal of the probe that has not hybridized to the target nucleic acid. For the detection, the target nucleic acid in the sample is preferably amplified to a detectable amount by a nucleic
15 acid amplification method such as PCR, NASBA or 3SR method, among which isothermal nucleic acid amplification methods such as the NASBA and 3SR methods are most preferable. When incorporating the nucleotide-labeled probe in the reaction solution during the amplification,
20 it is especially preferable to modify the probe by, for example, adding glycolic acid to the 3'-end so that the probe will not function as a nucleotide primer.

The invention of claim 5, which has been accomplished to achieve the second object, relates to a
25 detection method employing a RNA amplification process, which comprises the steps of: forming a cDNA with a RNA-dependent DNA polymerase using a specific sequence of a RNA derived from mecA gene, a gene element of MRSA, present in a sample as a template, with a first primer
30 having a sequence homologous to said specific sequence and a second primer having a sequence complementary to said specific sequence, wherein either the first or second primer has a sequence having the RNA polymerase promoter sequence added at its 5'-region, thereby
35 producing a RNA-DNA double-strand; digesting the RNA of said RNA-DNA double-strand with Ribonuclease H to form a single-stranded DNA; and then forming a double-stranded

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DNA that includes a promoter sequence allowing transcription of said RNA sequence or a RNA comprising a sequence complementary to said RNA sequence with a DNA-dependent DNA polymerase using said single-stranded DNA as a template, said double-stranded DNA produces a RNA transcription product in the presence of a RNA polymerase, and said RNA transcription product is subsequently used as the template for the single-stranded DNA production with said RNA-dependent DNA polymerase; characterized in that the oligonucleotide of SEQ. ID. No.18 is used as the first primer and the oligonucleotide of any of SEQ. ID. Nos.19 to 21 is used as the second primer, or the oligonucleotide of SEQ. ID. No.22 is used as the first primer and the oligonucleotide of SEQ. ID. Nos.23 or 24 is used as the second primer, or the oligonucleotide of SEQ. ID. No.25 is used as the first primer and the oligonucleotide of SEQ. ID. Nos.23 or 24 is used as the second primer.

The invention of claim 6, which has been accomplished to achieve the aforementioned object, relates to the detection method of claim 5, characterized in that said first primer is an oligonucleotide comprising at least 10 contiguous bases of the sequence of SEQ. ID. Nos.18, 22 or 25.

The invention of claim 7, which has been accomplished to achieve the aforementioned object, relates to the detection method of claim 5, characterized in that said second primer is an oligonucleotide comprising at least 10 contiguous bases of the sequence of SEQ. ID. Nos.19, 20, 21, 23 or 24.

The invention of claim 8, which has been accomplished to achieve the aforementioned object, relates to a detection method for a methicillin-resistant *Staphylococcus aureus* (MRSA), which comprises the steps of: conducting the RNA amplification process according to claim 5 in the presence of an oligonucleotide probe labeled with an intercalator fluorescent dye, wherein the

The present invention provides a combination of oligonucleotides for amplifying and detecting RNA derived from the *mecA* gene of methicillin-resistant *Staphylococcus aureus* at a relatively low and constant temperature (35-50°C, and preferably 41°C). Namely, the present invention provides a combination of an oligonucleotide primer for amplification of RNA derived from *mecA* gene and an oligonucleotide probe for detection and thereby provides a simple, rapidly and highly sensitive *mecA* gene detection method and a detection kit for clinical examination or the like using said combination.

combination.

20 In one mode for carrying out the present invention,
a second primer (a sequence complementary to the 3' end
region of a specific sequence of the target RNA)
complementary binds to the specific sequence of RNA
derived from mecA gene of methicillin-resistant
25 *Staphylococcus aureus* present in a sample as a template,
and cDNA is produced by an extension reaction with RNA-
dependent DNA polymerase to form a RNA-DNA double-strand,
after which the RNA of the RNA-DNA double-strand is
digested with Ribonuclease H to produce a single-stranded
30 DNA. Next, a first primer (a sequence homologous to the
5' end region of the target RNA, and including the RNA
polymerase promoter sequence added at the 5' end)
complementary binds to the single-stranded DNA, to
produce a double-stranded DNA having a promoter sequence
35 allowing transcription of RNA comprising a sequence
homologous to the target RNA sequence, using DNA-
dependent DNA polymerase. The double-stranded DNA is

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prevent it from functioning as a primer in the extension reaction. Examples of oligonucleotides that can be used for the oligonucleotide probe for detection include the sequences described in SEQ ID Nos. 20 or 29.

5 In an another mode for carrying out the present invention, a second primer (a sequence complementary to the target RNA, and including the RNA polymerase promoter sequence added at the 5' region) complementary binds to the specific sequence of RNA derived from the *mecA* gene of methicillin-resistant *Staphylococcus aureus* present in
10 a sample as a template, and cDNA is produced by extension reaction with RNA-dependent DNA polymerase to form a RNA-DNA double-strand, after which the RNA of the RNA-DNA double-strand is digested with Ribonuclease H to produce
15 a single-stranded DNA. Next, a first primer (a sequence homologous to the 5' end region of the target RNA) complementary binds to the single-stranded DNA, to produce a double-stranded DNA having a promoter allowing the transcription of RNA comprising a sequence
20 complementary to the target RNA sequence, using DNA-dependent DNA polymerase. The double-stranded DNA is then used for amplification of the RNA transcription product comprising the sequence complementary to the target RNA sequence in the presence of RNA polymerase.
25 The present invention is thus characterized by the use of an oligonucleotide of SEQ ID Nos.18, 22 or 25 for the first primer and an oligonucleotide of SEQ ID Nos.19, 20, 21, 23 or 24 for the second primer. Although the first and second primers may be the entire length of their
30 respective sequences, combinations of oligonucleotides comprising at least 10 contiguous bases within each sequence may also be used.

In the above mode of the present invention, the amplification process is preferably carried out in the
35 presence of an oligonucleotide probe (detecting oligonucleotide probe) labeled with an intercalator fluorescent dye having a sequence complementary to at

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Complementary binding of the probe to the RNA

complex formation is absent, so that the fluorescence

intensity of the reaction solution and
addition, when a labeled oligonucleotide probe is

example, addition of glycolic acid to the 3'-end, to

reaction. Examples of oligonucleotides for the oligonucleotide probe for detection include the

Brief Description of the Drawings

Fig. 1 is a photograph (black and

showing the status of an oligonucleotide,
illustrates an electrophoresis image of 7 M urea-5%

oligonucleotides designed for an intramolecular

this figure, lane M is the RNA marker, and lanes 1 through 25 are the numbers of the oligonucleotide

Fig. 2 illustrates the results of performing

primers in Example 2. In the figure, P represents the

copies/30 μ l is used, and N represents the number of copies of the target sequence. In addition, only diluent is used instead of RNA sample.

15 indicate the numbers of the combinations of p
probe in Example 2.

Fig. 3 illustrates the chemical structure

Fig. 4 illustrates the relationship between the reaction time and the fluorescence increasing rate. 1 and 2 represent the numbers of combinations of primers and probes in Example 3. In the graphs, for combination 1, P represents the case where a RNA sample of an initial RNA amount of 10^6 copies/30 μ l is used and, for combination 2, P represents the case where a RNA sample of an initial RNA amount of 10^4 copies/30 μ l is used. N represents the case where only diluent is used instead of RNA sample.

RNA sample.

Fig. 5 illustrates a graph showing an increase in the fluorescence increasing rate with respect to the reaction time and the formation of RNA at initial RNA amounts ranging from 10^4 copies/30 μ l to 10 copies/30 μ l as measured in Example 4. Nega indicates the use of only diluent instead of a RNA sample.

Examples

The present invention will now be explained in greater detail by way of examples, with the understanding that the invention is not limited by these examples.

Example 1
Specific binding of the oligonucleotides of the invention to *mecA*-RNA at 41°C was examined. The *mecA*-RNA is a synthesized and purified RNA obtained by *in vitro* transcription using double-stranded DNA containing the *mecA*-RNA base sequence as the template.

First, a sample of a standard RNA (2016mer) comprising base Nos. 1 to 2013 of *mecA*-RNA derived from PBP-2' (the RNA base sequence numbering is in accordance with Matsubashi, et al. FEBS Lett., 221, 167-171 (1987)) was quantified by ultraviolet absorption at 260 nm, and then diluted with an RNA diluent (10 mM Tris-HCl (pH

8.0), 0.1 mM EDTA, 0.5 U/ μ l RNase Inhibitor) to 2.0×10^{-12} mol/ μ l.

Next, the following compositions were dispensed into 0.5 ml volume PCR tubes (GeneAmp Thin-Walled ReactionTM Tubes; Perkin-Elmer Co., Ltd.).

- 5 0.90 μ l of 1 M Tris-HCl buffer (pH 8.6)
 0.20 μ l of 1 M magnesium chloride
 0.67 μ l of 2 M potassium chloride
 0.15 μ l of 0.1 M DTT
10 0.33 μ l of 119 U/ μ l RNase inhibitor
 9.95 μ l of distilled water
 0.6 μ l of 2 pmol/ μ l mecA-RNA sample
 1.2 μ l of 1.0 μ M oligonucleotide solution

15 In this context, one of the oligonucleotide solutions numbered below was used as the oligonucleotide solution.

1. Oligonucleotide complementary to base Nos.241 to 261
 of mecA-RNA (SEQ ID No. 1)
2. Oligonucleotide complementary to base Nos.264 to 283
 of mecA-RNA (SEQ ID No. 2)
20 3. Oligonucleotide complementary to base Nos.296 to 315
 of mecA-RNA (SEQ ID No. 3)
 4. Oligonucleotide complementary to base Nos.349 to 368
 of mecA-RNA (SEQ ID No. 4)
25 5. Oligonucleotide complementary to base Nos.402 to 421
 of mecA-RNA
 6. Oligonucleotide complementary to base Nos.425 to 444
 of mecA-RNA
 7. Oligonucleotide complementary to base Nos.456 to 475
 of mecA-RNA (SEQ ID No. 5)
30 8. Oligonucleotide complementary to base Nos.499 to 480
 of mecA-RNA
 9. Oligonucleotide complementary to base Nos.551 to 532
 of mecA-RNA (SEQ ID No. 6)

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10. Oligonucleotide complementary to base Nos.556 to 575
of mecA-RNA (SEQ ID No. 7)
11. Oligonucleotide complementary to base Nos.581 to 600
of mecA-RNA (oligonucleotide of the 16th to 35th
bases from the 5' end of the sequence shown in SEQ
ID No.8)
12. Oligonucleotide complementary to base Nos.606 to 625
of mecA-RNA
13. Oligonucleotide complementary to base Nos.672 to 691
of mecA-RNA (SEQ ID No. 9)
14. Oligonucleotide complementary to base Nos.941 to 961
of mecA-RNA (SEQ ID No. 10)
15. Oligonucleotide complementary to base Nos.967 to 986
of mecA-RNA (SEQ ID No. 11)
16. Oligonucleotide complementary to base Nos.1134 to
1153 of mecA-RNA (SEQ ID No. 12)
17. Oligonucleotide complementary to base Nos.1154 to
1173 of mecA-RNA (SEQ ID No. 13)
18. Oligonucleotide complementary to base Nos.1221 to
1240 of mecA-RNA (SEQ ID No. 14)
19. Oligonucleotide complementary to base Nos.1656 to
1675 of mecA-RNA (SEQ ID No. 15)
20. Oligonucleotide complementary to base Nos.1701 to
1720 of mecA-RNA (SEQ ID No. 16)
21. Oligonucleotide complementary to base Nos.1852 to
1871 of mecA-RNA
22. Oligonucleotide complementary to base Nos.1906 to
1925 of mecA-RNA
23. Oligonucleotide complementary to base Nos.596 to 615
of mecA-RNA (oligonucleotide of the 1st to 20 the
bases from the 5' end of the sequence shown in SEQ
ID No. 8)
24. Oligonucleotide complementary to base Nos.577 to 615
of mecA-RNA (SEQ ID No. 8)
25. Oligonucleotide complementary to base Nos.1087 to
1100 of mecA-RNA (SEQ ID No. 17)
- The reaction solutions were then incubated at 41°C

for 5 minutes, 1 μ l of AMV-Rtase(Takara Shuzo Co., Ltd.; AMV-Rtase is an enzyme that cleaves the RNA of DNA/RNA double strands) was added, and the PCR tube was incubated at 41°C for 15 minutes.

5 Polyacrylamide gel (acrylamide concentration: 5%, urea: 7 M) electrophoresis was conducted to confirm the cleaved fragments after the reaction. Dyeing after electrophoresis was carried out with a commercially available dye (SYBR Green II™ (Takara Shuzo Co., Ltd.)).
10 Upon binding of the oligonucleotide to the specific site of the target RNA, the RNA of the DNA/RNA double strands is cleaved by the ribonuclease H activity of AMV-Rtase, which allows observation of specific bands.

The results of electrophoresis are shown in Fig. 1.
15 Regarding the bands newly appearing in each lane, in the lanes where two bands could be observed as a result of a specific cleavage with one of the employed oligonucleotide, bands with shorter length are indicated with arrows. In addition, the bands exhibiting
20 significant non-specific cleavage are encircled. Among the above oligonucleotides, only oligonucleotide solutions containing SEQ ID Nos. 1 through 17 and SEQ ID No. 8 showed characteristic cleaved bands without
25 significant non-specific cleavage, demonstrating that each of these oligonucleotides strongly binds to mecA-RNA at 41°C.

Results

As explained above, the oligonucleotides of the present invention are oligonucleotides that complementary
30 bind to RNA derived from mecA-gene coding for PBP-2', even under conditions of relatively low and constant temperature (35-50°C, preferably 41°C), which tend to produce an intramolecular structure in RNA and prevent binding of primers and probes thereto. Specific binding
35 of the oligonucleotides is therefore possible without heat denaturation of the target RNA. The oligonucleotides of the invention are thus useful as

5 Furthermore, the oligonucleotides of the invention are also clearly useful for amplification and detection of the *mecA* gene. Oligonucleotides complementary to the above-mentioned oligonucleotides are also useful for amplification of double-stranded DNA by the PCR method, or for detection of cDNA obtained by reverse transcription of RNA.

The oligonucleotides of the invention are not limited to the specifically listed base sequences (20mers), and they may be oligonucleotides comprising at least 10 or more contiguous bases within those sequences. This is obvious from the fact that 10mer base sequences are sufficient to ensure adequate specificity of primers or probes to target nucleic acids under relatively low temperature condition (preferably, at 41°C).

Specific amplification of target RNA was carried out using a combination of oligonucleotide primers according to the present invention. The mecA-RNA is a synthesized and purified RNA obtained by *in vitro* transcription using double-stranded DNA containing the mecA-RNA base sequence as the template.

(1) A sample of a standard RNA (2016mer) comprising base Nos. 1 to 2013 of *mecA*-RNA derived from PBP-2' (the RNA base sequence numbering is in accordance with Matsubashi, et al. FEBS Lett., 221, 167-171 (1987)) was quantified by ultraviolet absorption at 260 nm, and then diluted with an RNA diluent (10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 0.5 U/ μ l RNase Inhibitor, 5.0mM DTT) to 1.0×10^4 copies/2.5 μ l.

In the control testing group, only the diluent was used

(Nega).

- (2) 23.3 μ l of a reaction liquid having the composition indicated below was dispensed into 0.5 ml volume PCR tubes (Gene Amp Thin-Walled Reaction Tubes, Perkin-Elmer) followed by addition of 2.5 μ l of the above RNA sample.

Composition of Reaction Liquid (concentrations refer to the final concentrations in the reaction system following addition of enzyme solution)

- 60.0 mM Tris-HCl buffer (pH 8.6)
- 13.0 mM magnesium chloride
- 90.0 mM potassium chloride
- 1.0 mM DTT
- 0.25 mM each of dATP, dCTP, dGTP and dTTP
- 3.0 mM each of ATP, GTP and UTP
- 2.25 mM GTP
- 3.6 mM ITP
- 1.0 μ M each of the first and second primers
- 0.16 μ M of a cleaving oligonucleotide probe (oligonucleotide for cleaving the target RNA at a position to which the first primer is capable to bind; the 3' end thereof is aminated)
- 39 U ribonuclease inhibitor (Takara Shuzo)
- 15.0% DMSO
- Distilled water for adjusting volume

One of the combinations numbered below was used for the combination of the first primer, the second primer and the cleaving probe.

1. As for the first primer, oligonucleotide of the 4th to 15th bases from the 5' end of the sequence shown in SEQ ID No.18, wherein the promoter sequence of T7 polymerase shown in SEQ ID No.30 is added to its 5' end; as for the second primer, the oligonucleotide shown in SEQ ID No.19; and as for the cleaving probe, the oligonucleotide shown in SEQ ID No.26.
2. As for the first primer, oligonucleotide of the 4th to 15th bases from the 5' end of the sequence shown

in SEQ ID No.18, wherein the promoter sequence of T7 polymerase shown in SEQ ID No.30 is added to its 5' end; as for the second primer, the oligonucleotide shown in SEQ ID No.20; and as for the cleaving probe, the oligonucleotide shown in SEQ ID No.26.

3. As for the first primer, oligonucleotide of the 4th to 15th bases from the 5' end of the sequence shown in SEQ ID No.18, wherein the promoter sequence of T7 polymerase shown in SEQ ID No.30 is added to its 5' end; as for the second primer, the oligonucleotide shown in SEQ ID No.21; and as for the cleaving probe, the oligonucleotide shown in SEQ ID No.26.
4. As for the first primer, oligonucleotide of the 4th to 28th bases from the 5' end of the sequence shown in SEQ ID No.18, wherein the promoter sequence of T7 polymerase shown in SEQ ID No.30 is added to its 5' end; as for the second primer, the oligonucleotide shown in SEQ ID No.20; and as for the cleaving probe, the oligonucleotide shown in SEQ ID No.26.
5. As for the first primer, oligonucleotide of the 4th to 28th bases from the 5' end of the sequence shown in SEQ ID No.18, wherein the promoter sequence of T7 polymerase shown in SEQ ID No.30 is added to its 5' end; as for the second primer, the oligonucleotide shown in SEQ ID No.21; and as for the cleaving probe, the oligonucleotide shown in SEQ ID No.26.
6. As for the first primer, oligonucleotide of the 1st to 25th bases from the 5' end of the sequence shown in SEQ ID No.18, wherein the promoter sequence of T7 polymerase shown in SEQ ID No.30 is added to its 5' end; as for the second primer, the oligonucleotide shown in SEQ ID No.20; and as for the cleaving probe, the oligonucleotide shown in SEQ ID No.26.
7. As for the first primer, oligonucleotide of the 1st to 25th bases from the 5' end of the sequence shown in SEQ ID No.18, wherein the promoter sequence of T7 polymerase shown in SEQ ID No.30 is added to its 5'

end; as for the second primer, the oligonucleotide shown in SEQ ID No.21; and as for the cleaving probe, the oligonucleotide shown in SEQ ID No.26.

- 5 8. As for the first primer, oligonucleotide of the 4th to 28th bases from the 5' end of the sequence shown in SEQ ID No.22, wherein the promoter sequence of T7 polymerase shown in SEQ ID No.30 is added to its 5' end; as for the second primer, the oligonucleotide shown in SEQ ID No.23; and as for the cleaving
10 probe, the oligonucleotide shown in SEQ ID No.27.
- 15 9. As for the first primer, oligonucleotide of the 4th to 28th bases from the 5' end of the sequence shown in SEQ ID No.22, wherein the promoter sequence of T7 polymerase shown in SEQ ID No.30 is added to its 5' end; as for the second primer, the oligonucleotide shown in SEQ ID No.24; and as for the cleaving
20 probe, the oligonucleotide shown in SEQ ID No.27.
- 25 10. As for the first primer, oligonucleotide of the 1st to 25th bases from the 5' end of the sequence shown in SEQ ID No.22, wherein the promoter sequence of T7 polymerase shown in SEQ ID No.30 is added to its 5' end; as for the second primer, the oligonucleotide shown in SEQ ID No.23; and as for the cleaving
30 probe, the oligonucleotide shown in SEQ ID No.27.
- 35 11. As for the first primer, oligonucleotide of the 1st to 25th bases from the 5' end of the sequence shown in SEQ ID No.22, wherein the promoter sequence of T7 polymerase shown in SEQ ID No.30 is added to its 5' end; as for the second primer, the oligonucleotide shown in SEQ ID No.24; and as for the cleaving
30 probe, the oligonucleotide shown in SEQ ID No.27.
12. As for the first primer, oligonucleotide of the 4th to 28th bases from the 5' end of the sequence shown in SEQ ID No.25, wherein the promoter sequence of T7 polymerase shown in SEQ ID No.30 is added to its 5' end; as for the second primer, the oligonucleotide shown in SEQ ID No.23; and as for the cleaving

probe, the oligonucleotide shown in SEQ ID No.28.

13. As for the first primer, oligonucleotide of the 4th to 28th bases from the 5' end of the sequence shown in SEQ ID No.25, wherein the promoter sequence of T7 polymerase shown in SEQ ID No.30 is added to its 5' end; as for the second primer, the oligonucleotide shown in SEQ ID No.24; and as for the cleaving probe, the oligonucleotide shown in SEQ ID No.28.

14. As for the first primer, oligonucleotide of the 1st to 25th bases from the 5' end of the sequence shown in SEQ ID No.25, wherein the promoter sequence of T7 polymerase shown in SEQ ID No.30 is added to its 5' end; as for the second primer, the oligonucleotide shown in SEQ ID No.23; and as for the cleaving probe, the oligonucleotide shown in SEQ ID No.28.

15. As for the first primer, oligonucleotide of the 1st to 25th bases from the 5' end of the sequence shown in SEQ ID No.25, wherein the promoter sequence of T7 polymerase shown in SEQ ID No.30 is added to its 5' end; as for the second primer, the oligonucleotide shown in SEQ ID No.24; and as for the cleaving probe, the oligonucleotide shown in SEQ ID No.28.

(3) After incubating the above reaction solutions for 5 minutes at 41°C, 4.2 µl of enzyme liquid having the following composition and pre-incubated for 2 minutes at 41°C was added.

Composition of Enzyme Liquid (final concentrations during reaction)

1.7% sorbitol

8 units of AMV reverse transcriptase (Takara Shuzo)

142 units of T7 RNA polymerase (Gibco)

3 µg of bovine serum albumin

Distilled water for adjusting volume

(4) After which, the PCR tubes were incubated for 90 minutes at 41°C, and then the specific amplification products were analyzed by electrophoresis using 4%

agarose gel.

(5) A commercially available dye (SYBR Green II™: Takara Shuzo) was used for staining after electrophoresis.

5 The results of electrophoresis are shown in Fig. 2 (black and white inverted photograph). For all of the combinations, specific RNA amplification products (indicated with arrows) were obtained in the systems to which *mecA*-RNA was added. On the basis of this finding, 10 these combinations of oligonucleotide primers were demonstrated as being useful in the amplification and detection of RNA derived from the *mecA* gene of methicillin-resistant *Staphylococcus aureus*.

Example 3

15 Using the combination of oligonucleotide primers according to the present invention, the possibility of specific detection of *mecA*-RNA, the target RNA, was confirmed.

20 (1) A sample of a standard RNA (2016mer) comprising base Nos. 1 to 2013 of *mecA*-RNA derived from PBP-2' (the RNA base sequence numbering is in accordance with Matsubashi, et al. FEBS Lett., 221, 167-171 (1987)) was quantified by ultraviolet absorption at 260 nm, and then diluted with an RNA diluent (10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 0.5 25 U/μl RNase Inhibitor, 0.5mM DTT) to 1.0×10^6 copies/2.5μl or 1.0×10^4 copies/2.5μl In the control testing group, only the diluent was used (Nega).

30 (2) 23.3 μl of a reaction liquid having the composition indicated below was dispensed into 0.5 ml volume PCR tubes (Gene Amp Thin-Walled Reaction Tubes, Perkin-Elmer) followed by addition of 2.5 μl of the above RNA sample (*mecA*-RNA).

35 Composition of Reaction Liquid (concentrations refer to the final concentrations in the reaction system following addition of enzyme solution)

60.0 mM Tris-HCl buffer (pH 8.6)

- 13.0 mM magnesium chloride
90.0 mM potassium chloride
1.0 mM DTT
0.25 mM each of dATP, dCTP, dGTP and dTTP
5 3.0 mM each of ATP, GTP and UTP
2.25 mM GTP
3.6 mM ITP
1.0 μ M of the first oligonucleotide primer
1.0 μ M of the second oligonucleotide primer
10 0.16 μ M cleaving oligonucleotide probe
(oligonucleotide for cleaving the target RNA at a
position to which the first primer is capable to bind;
its 3' end is aminated)
25.0 nM of oligonucleotide probe for detection
15 labeled with intercalator fluorescent dye(Fig. 3) (MRSH-
YO; its 3' end modified with glycolic acid)
39 U ribonuclease inhibitor (Takara Shuzo)
15.0% DMSO
Distilled water for adjusting volume
20 One of the combinations numbered below was used for
the combination of primers and probe.
1. As for the first primer, oligonucleotide of the 4th
to 15th bases from the 5' end of the sequence shown
in SEQ ID No.18, wherein the promoter sequence of T7
25 polymerase shown in SEQ ID No.30 is added to its 5'
end; as for the second primer, the oligonucleotide
shown in SEQ ID No.19; as for the cleaving probe,
the oligonucleotide shown in SEQ ID No.26, and as
for the probe for detection, the oligonucleotide
30 shown in SEQ ID No.20.
2. As for the first primer, oligonucleotide of the 1st
to 25th bases from the 5' end of the sequence shown
in SEQ ID No.25, wherein the promoter sequence of T7
polymerase shown in SEQ ID No.30 is added to its 5'
35 end; as for the second primer, the oligonucleotide
shown in SEQ ID No.24; as for the cleaving probe,

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the oligonucleotide shown in SEQ ID No.28; and as for the detecting probe, the oligonucleotide shown in SEQ ID No.29.

- 5 (3) After incubating the above reaction solution for 4 minutes at 41°C, 4.2 µl of enzyme liquid having the following composition and pre-incubated for 2 minutes at 41°C were added.

Composition of Enzyme Liquid (final concentrations during reaction)

- 10 1.7% sorbitol
8 units of AMV reverse transcriptase (Takara Shuzo)
142 units of T7 RNA polymerase (Gibco)
3 µg of bovine serum albumin
Distilled water for adjusting volume

- 15 (4) Next, using a temperature-controllable fluorescent spectrophotometer capable of directly measuring PCR tubes, periodic measurement of the fluorescence intensity of the reaction solution incubated at 41°C with an excitation wavelength of 470 nm and a fluorescent wavelength of 510 nm was carried out. Fig. 4 shows the
20 periodic changes in the fluorescence intensity ratio (fluorescence intensity at predetermined time/background fluorescence intensity) of the sample, where enzyme was added at 0 minutes. The RNA sample concentrations were, for combination 1, 10⁶ copies/30 µl, and for combination
25 2, 10⁴ copies/30 µl.

- In the system in which mecA-RNA was added to targeted RNA, specific fluorescent sensitization was obtained. On the basis of this finding, the combination
30 of oligonucleotides of the present invention were demonstrated as being capable to specifically amplify and detect RNA derived from mecA gene.

Example 4

- 35 A combination of oligonucleotide primers according to the invention was used for specific detection of different initial copy numbers of the target RNA.

(1) A sample of a standard RNA (2016mer) comprising base Nos. 1 to 2013 of mecA-RNA derived from PBP-2' (the RNA base sequence numbering is in accordance with Matsubashi, et al. FEBS Lett., 221, 167-171 (1987)) was quantified by ultraviolet absorption at 260 nm, and then diluted with an RNA diluent (10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 0.5 U/ μ l RNase Inhibitor, 5.0mM DTT) to concentrations ranging from 1.0×10^4 copies/2.5 μ l to 10 copies/2.5 μ l. In the control testing group, only the diluent was used (Nega).

(2) 23.3 μ l of a reaction liquid having the composition indicated below was dispensed into 0.5 ml volume PCR tubes (Gene Amp Thin-Walled Reaction Tubes, Perkin-Elmer) followed by addition of 2.5 μ l of the above RNA sample (mecA-RNA).

Composition of Reaction Liquid (concentrations refer to the final concentrations in the reaction system following addition of enzyme solution)

60.0 mM Tris-HCl buffer (pH 8.6)
13.0 mM magnesium chloride
90.0 mM potassium chloride
1.0 mM DTT
0.25 mM each of dATP, dCTP, dGTP and dTTP
3.0 mM each of ATP, CTP and UTP
2.25 mM GTP
3.6 mM ITP

1.0 μ M of the first primer (oligonucleotide of the 4th to 28th bases from the 5' end of the sequence shown in SEQ ID No.25, wherein the promoter sequence of T7 polymerase shown in SEQ ID No.30 is added to its 5' end

1.0 μ M of the second oligonucleotide primer (oligonucleotide of the 1st to 18th bases of from 5' end of the sequence shown in SEQ ID No.23)

0.16 μ M cleaving oligonucleotide probe (SEQ ID No.28: oligonucleotide for cleaving the target RNA at a

position to which the first primer is able to bind,
wherein its 3' end is aminated)

25.0 nM of oligonucleotide probe for detection
labeled with intercalator fluorescent pigment (Fig. 3)
5 (MRSH-YO; its oligonucleotide sequence is shown in SEQ ID
No.29, and its 3' end is modified with glycolic acid)

39 U ribonuclease inhibitor (Takara Shuzo)

15.0% DMSO

Distilled water for adjusting volume

10 (3) After incubating the above reaction solution for 4
minutes at 41°C, 4.2 µl of enzyme liquid having the
following composition and pre-incubated for 2 minutes at
41°C were added.

Composition of Enzyme Liquid (final concentrations
15 during reaction)

1.7% sorbitol

8 units of AMV reverse transcriptase (Takara Shuzo)

142 units of T7 RNA polymerase (Gibco)

3 µg of bovine serum albumin

20 Distilled water for adjusting volume

(4) Next, using a temperature-controllable fluorescent
spectrophotometer capable of directly measuring PCR
tubes, periodic measurement of the fluorescence intensity
of the reaction solution incubated at 41°C with an
25 excitation wavelength of 470 nm and a fluorescent
wavelength of 510 nm was carried out. Fig. 5 shows the
periodic changes in the fluorescence intensity ratio
(fluorescence intensity at predetermined time/background
fluorescence intensity) of the sample, where enzyme was
30 added at 0 minutes. The RNA sample concentrations were
10 copies/30 µl to 10⁴ copies/30 µl.

A fluorescence profile depending on the initial
concentration of the target RNA was obtained from Fig. 5,
indicating that it is possible to measure the amount of
35 RNA derived from the *mecA* gene present in unknown
samples.

Results

As has been explained above, the present invention is useful as combinations of oligonucleotide primers and oligonucleotide probes which specifically bind to RNA
5 derived from the *mecA* gene coding for PBP-2', and rapidly amplify and detect the target RNA even under relatively low and constant temperature (35-50°C and preferably 41°C) conditions in which RNA in a sample would form an intramolecular structure which inhibits the primer and
10 probe binding.

In addition to the above, the combinations of oligonucleotides of the present invention are not only useful for *mecA*-RNA, but also as complementary sequences of the above oligonucleotides for detecting cDNA obtained
15 by reverse transcription of RNA.

The base lengths of the oligonucleotides in the combinations of the present invention are not limited to the concretely described lengths, but rather include oligonucleotides comprised of at least 10 contiguous
20 bases within these sequences. This is clear from the fact that a base sequence of about 10 mer is adequate for ensuring specificity of primer or probe to a target nucleic acid under relatively low temperature (preferably 41°C) conditions.

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